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13. ABSTRACT ***(Maximum 200 Words)***
The long term goal of this research program is to develop fibroblast growth factor-1 (FGF-1) through rational protein engineering into a potent and specific anabolic agent for the treatment of osteoporosis and fracture repair. The specific aims of this research plan remain:

1) to evaluate the effects of existing mutant forms of FGF-1 on bone cells *in vitro*, on bone formation *in vivo*, and to assess their toxicological or undesirable activities
2) to generate additional FGF-1 mutants or chimeric proteins that are likely to exhibit enhanced anabolic activity on bone with reduced toxicological effects.

During the current year of support we have made significant progress with regard to these specific aims. The most important finding was the identification and production of FGF-1 variants with enhanced anabolic activity for bone. We also identified differences in the abilities of these constructs to promote osteoblast differentiation from marrow cultures. Finally we have demonstrated for the first time the ability of an FGF-1 variant to exhibit increased activity for bone with reduced hypotensive effects relative to the wild-type protein.

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William H. Burgess 10/99

PI - Signature Date

Table of Contents

Introduction

The long term goal of this research program remains to develop fibroblast growth factor-1 (FGF-1) via protein engineering into a potent and specific anabolic agent for the treatment of osteoporosis and fracture repair. Osteoporosis is a disease which afflicts nearly 200 million people worldwide and this number is expected to double in the next 20-30 years. It is likely that all people with the disease would benefit from treatments to increase bone mass. The greatest therapeutic challenge in the osteoporosis field at the present time is the identification of an agent that promotes significant new bone formation. Although there are effective resorption inhibitors for the treatment of osteoporosis (bisphosphanates, estrogen, and calcitonin) these drugs essentially stabilize bone mass but do not lead to substantial increases in bone mass or the restoration of trabecular bone microarchitecture. For patients with severe and established osteoporosis, there is a tremendous need for therapeutic agents that stimulate bone formation and initiate the cascade of events involved in osteoblast differentiation. Those agents that are known to have a stimulatory effect on new bone formation are fluoride, low-dose intermittent parathyroid hormone and its analogs, and the peptide growth factors that are incorporated into the bone matrix and released from bone as it resorbs.

During the past several years, it has become apparent that members of the FGF family of ligands and receptors are essential for normal skeletal growth (1). The preliminary data that formed the basis of the original application demonstrated a significant osteogenic potential for local and systemic administration of FGF-1 *in vivo*. The data also documented certain toxicological or undesirable effects associated with these treatments. Together these data indicated that the therapeutic window is relatively narrow. In the progress report for the first year of funding, we outlined our progress on the generation of several mutants of FGF-1 and chimeric proteins. We also reported on the *in vitro* and *in vivo* activities of these proteins. The studies confirmed that a further evaluation of existing mutants and production of additional mutants or chimeric proteins may improve the efficacy of FGF-1 as an anabolic factor in the treatment of osteoporosis.

The original specific aims of the proposal were:

1) To evaluate the effects of existing mutant forms of FGF-1 on bone cells *in vitro*, on bone formation *in vivo* and to assess their toxicological or undesirable effects.

2) To generate additional FGF-1 mutants or chimeric proteins that are likely to exhibit enhanced anabolic activity on bone with reduced toxicological effects.

Progress towards these aims achieved during the previous and current funding period are summarized in the following report.

Body

In the progress report for the previous year of support, we described our studies of the cys-free mutant of FGF-1. We demonstrated that this mutant was ~10 fold more active than wild-type FGF-1 in restoring trabecular bone mass in aged ovariectomized rats. In addition, we provided histological evidence that the cys-free mutant had reduced toxicological effects on renal glonerular capillaries and basement membranes. This represented the first data to indicate that FGF-1 could be modified through site-directed mutagenesis to exhibit enhanced anabolic activity for bone with reduced undesirable side effects. Together this data validated the hypothesis of the original application. During the current year of support we have developed several important *in vitro* assays for evaluation of different mutants, initiated blood pressure studies and evaluated several new FGF-1 constructs for their anabolic effects on bone. In addition we have established the capacity to study non-demineralized sections of bone. This allows us to make a better assessment of the quality of the new bone formation induced by systemic FGF-1 therapy in the ovariectomized rat model of osteoporosis. These results are summarized below.

New FGF-1 Constructs: Two new FGF-1 constructs were produced during the current year of support. One is a chimeric molecule consisting of the protein HB-GAM (heparin binding growth associated molecule) fused in frame with wild-type FGF-1. We and others have shown that HB-GAM is not a mitogen for a variety of cell lines (2). It has been reported to be found at relatively high concentrations in bone and to be a chemoatractant for osteoblastic cells (3). In addition over-expression of the protein in transgenic animals results in increased thickness of cortical bones. We expressed the chimeric protein with the thought that the HB-GAM portion would target FGF-1 more selectively to bone. Studies performed to date with the purified chimera are described in subsequent sections. The second construct referred to as FGF-1 678, has a serine to lysine substitution at position 130. We have shown that the region of FGF-1 is a major heparin-binding domain (4). In addition when basic residues in this region are replaced by mutagenesis

with non-basic amino acids, the protein exhibits reduced heparin affinity and reduced mitogenic activity (5). The 678 mutation represents an attempt to increase mitogenic potency through the addition of extra basic amino acids in the region.

Effects of FGF-1 Constructs on Bone Marrow Differentiation: Two six week old male SD rats were sacrificed by CO_2 asphyxiation and their femora and tibia removed and cleaned of fibrous tissue. The ends of the bones were removed and the marrow flushed out, using a syringe and 18G needle, with 5-10ml alphaMEM. The marrow cells were pelleted by centrifugation (5 min at 800G), the supernatant removed, the cells resuspended in 10ml alphaMEM then filtered through a 70μm nylon mesh filter. After being counted, the cells were seeded into 6-well plates at a density of 10^5 cells/cm^2 (10^6 cells per well) in 3.5ml alphaMEM+10%FCS containing supplements to induce osteoblastic colony formation (100μM ascorbate-2-phosphate and 10nM dexamethasone). Each 6-well plate included the addition of one of the FGF-1 preparations (WT/CysFree or Chimera at 20ng/ml) +/- 5U/ml heparin.

The medium was replaced after 4 days, which removed any non-adherent cells, and thereafter every 3/4 days, each time the medium was replaced the growth factors and osteoblast-inducing factors were included. On the 18th day after seeding 10mMβ-glycerophosphate was added to the medium to allow mineralization to take place, and on the 21st day the colonies which had formed were fixed and stained for the presence of bone-related markers (alkaline phosphatase and mineralization).

The medium was removed from the wells, and PBS added to rinse the cells, this was removed and the colonies fixed in neutral buffered 10% formalin for 15 minutes at 4°C. The fixative was removed, and the colonies washed in dH_2O (1x1 min and 1x15 min). After removal of the second wash, AP substrate was added to each well (to 99.6 ml and 0.1M Tris-HCl pH8.3, add 10mg naphthol-AS-MX-phosphate dissolved in 400μl DMSO, mix well and add 40mg Fast Red Violot LB salt then filter to remove insoluble dye), and was incubated at room temperature for 40 minutes (an insoluble red dye will develop in the alkaline phosphatase positive colonies). After incubation, the substrate was removed and the colonies rinsed in tap water, this was followed by addition of Von Kossa's reagent to each well (2.5 silver nitrate in distilled water)

and incubation at room temperature for 30 minutes (mineralized areas of the colonies will turn black). The Von Kossa stain was then removed and the colonies rinsed twice in distilled water, this was followed by brief counterstaining with toluidine blue (0.1%w/v in 30%v/v ethanol) then two further rinses with distilled water, after which the stained colonies were allowed to dry. The results of one experiment are summarized in Figure 1. The figure shows the marrow cells after alkaline phosphatase (red) and Von Kossa staining for mineralized modules. The cells were treated with the indicated proteins in the presence or absence of 5U/ml heparin. Several points can be made from these studies. First, although heparin is known to potentiate the mitogenic activity of FGF-1, it clearly inhibits the formation of mineralized nodules in this assay. We assume the heparin inhibits the differentiation of the osteoblastic lineage from the marrow culture. The observation is particularly interesting given the large amount of literature on heparin induced osteoporosis. It is also clear that wild-type (WT) FGF-1 and the chimeric protein produce a significant increase in mineralized nodule formation whereas the cys free mutant of FGF-1 appears to inhibit. We showed previously that the cys-free mutant was ~10 fold more potent than wild-type in promoting mitogenesis of osteoblastic cells *in vitro* and producing new bone when injected locally over the calvaria. Although the cys-free is able to restore bone mass when given systemically, it does not appear to be 10 fold more potent in the osteoporosis model. This may be due to the increased mitogenic potency being off set by an inhibition of the formation of new osteoblastic cells from marrow stem cells. We believe the marrow culture is an important addition to our mutant analysis and that the differences seen among the different forms of FGF-1 is an important and exciting discovery.

Osteoblastic Migration and Attachment: We evaluated the ability of the chimeric FGF-1 to promote the migration and localization of human osteoblastic cells. The migration assays were performed using 24-well transwell filters (6mm diameter, 8μm pore size). Cells (MG-63) were trypsinized from stock plates, allowed to recover for 1 ½ hours in DMEM + 10% FCS prior to being seeded in the upper well at $1x10^5$ cells/cm^2 in DMEM. The lower wells contained wild-type FGF-1, FGF-1 chimera or BSA in DMEM. After 4 hours at 37°C in a CO^2 incubator the filters were removed, fixed in formalin and stained with 0.05% toluidine blue. The results of one such assay are shown in Figure 2. Panel A shows the BSA control. The arrow points to one of the pores in the filter. Panels B and C show the migration of cells through the filter in response

to FGF-1 (100ng/ml) and FGF-1 chimera (Panel C, 100ng/ml). In these panels the arrows point to migrated cells. Clearly the chimeric FGF-1 promotes increased migration of the osteoblastic cells. We also evaluated the chimeric protein as an attachment factor for osteoblastic cells. The chimera was pipetted in a line on tissue culture plastic, allowed to dry and washed. Osteoblastic cells were then added to the dish and allowed to attach overnight. Panel D of Figure 2 shows the boundary of the chimeric coating and reveals a tremendous increase in cell density on the coated portion (arrows point to boundary). Together these data demonstrate that the FGF-1 chimera is a potent chemoattractant and attachment factor for osteoblasts. It is not yet known whether the construct will target better to bone following systemic administration. It should effectively localize the growth factor to bone when applied locally.

Effects of Systemic Injection of Mutant Forms of FGF-1: Rats are ovariectomized, aged for 5 months to allow bone loss then started on daily tail vein injections of FGF-1 or modified forms for 28 days. The bone mass is allowed to stabilize for an additional 28 days without injections. The rats are then euthanized and the tibia, fibula and femurs are removed for histological analysis. In the past we demineralized tissue prior to sectioning and staining. This year we obtained an additional microtome (institutional funds) equipped with a tungstun/carbide knife that allows us to section mineralized bone. Tissue is cleaned from the bone then the bone is fixed in 70%, 95% and 100% ethanol for 48 hours each. The tissue is then infiltrated with plasticizer for two days then embedded at 37° C for 24 hours. 5 micron sections are cut and mineralized bone is visualized with Von Kossa silver stain. A summary of results is presented in Figure 3. The figure shows Von Kossa stained sections through the top of the tibia. Bone from a normal rat (NR1) and an untreated ovariectomized rat (OVX2) are shown at the top of the figure. The orange arrow points to well mineralized trabecular bone in the epiphyses (above growth slate) that is lost following ovariectomy. The orange arrows also point to regions in the treated animals (20μg/kg/day of wild-type, chimeric, cys free and the 678 mutated forms of FGF-1) where a dramatic restoration of mineralized bone mass was achieved. The green arrow point to regions where trabecular bone was clearly restored in the shaft. The tissues were subjected to quantitative analysis using the Bioquant Image Analysis system. The % mineralized tissue per high power field across the growth plate were; normal rat – 19.6%; ovx untreated – 5.4%; wild-type FGF-1 – 10.9%; chimeric FGF-1 – 13.7%; cys free FGF-1 – 14.8%; and the 678 mutant

20.5%. The key issue for the next year of support is to determine what combinations of these established forms can be produced and whether the multiple changes show enhanced anabolic activity for bone.

Effects of FGF-1 on Blood Pressure: Our last progress report was criticized for not having monitored the blood pressure effects of the various FGF-1 constructs. To some extent this was deliberate in that the studies are technically difficult and we have yet to settle on the most optimal mutant. We have begun these studies with a collaborator, Dr. Gregory Mundy. Phenobarbital sodium is used as an anesthetic and is given intraperitoneally as a bolus (50mg/kg), followed by intravenous infusion (88μg/min) in isotonic saline at 7μl/min. An IVC cannula is placed in the lateral cervical ventricle and a bladder catheter is implanted. Mean arterial blood pressure, systolic blood pressure, diastolic blood pressure and heart rate are determined following cannulation of the femora artery. The proteins are given as a bolus infusion. The results of two such studies are provided in Figures 4 and 5. At 30μg/kg both the wild-type and the cys-free mutant of FGF-1 resulted ~25% drop in systolic pressure (Figure 4). In contrast, at 5μg/kg the wild-type protein caused the same drop in pressure whereas the cys-free did not. These data demonstrate the concept of the original application is valid and the goals may be obtainable. We hope that future testing will be more routine. Towards this end we have used institutional funds to purchase a K18 5 cuff rat tail blood pressure system from Harvard Apparatus (~$5,000). The use of this system will result in reduced animal usage and we have sufficient baseline data to evaluate its performance in establishing the relative hypotensive effects of the various FGF-1 constructs.

Key Research Accomplishments

- Produced two new FGF-1 variants
- Established osteoblast migration and attachment assays
- Established stem cell differentiation assays
- Identified FGF-1 variants with enhanced anabolic activity for bone in osteoporosis model
- Established histological analysis of mineralized sections
- Demonstrated reduced hypotensive activity of cys-free mutant

Reportable Outcomes

- Tissue Engineering/Regenerative Healing/Stem Cell Biology Conference
  Cambridge Healthtech Institute, Pittsburgh, Pennsylvania
  Title: Fibroblast Growth Factor-1: Multiple Aspects of Bone Formation.
- D.J. Mackenzie, R. Sipe, D. Buck, W. Burgess, J. Hollinger. "Recombinant Human Acid Fibroblast Growth Factor and Fibrin Carrier Regenerate Bone." *J. Orthop. Res.* Submitted (1999).

Conclusions

Together, the data summarized in the body of this report and presented in previous progress reports demonstrate that the original hypothesis and statement of work were of merit and realistic. In the current progress report we summarize a large amount of work that could not have been anticipated in the original application due to technical limitations of the laboratory. We have identified three modifications of FGF-1 that show promise as improved anabolics for bone in a variety of *in vitro* and *in vivo* assays. The challenge for the final year of this application will be to identify the combinations of these modifications that result in the optimal growth factor for the rat osteoporosis model. Ultimately we must move the studies to a higher vertebrate species before we can consider studies in humans.

References

1. Y. Li, K. Mangasarian, A. Mansukhani, C. Basilico. *Oncogene.* **14**, 1397 (1997).
2. B.S. Hampton, D.R. Marshak, and W.H. Burgess. *Molec. Biol. Cell.* **3**, 85 (1992).
3. S. Imai, M. Kaksonen, E. Raulo, T. Kinnunen, C. Fages, X. Meng, M. Lakso, H. Rauvala. *J. Cell Biol.* **143**, 1113 (1998).
4. P. Wong, B. Hampton, E. Szylobryt, A.M. Gallagher, M. Jaye, W.H. Burgess. *J. Biol. Chem.* **270**, 25805 (1995).
5. W.H. Burgess, A.M. Shaheen, M. Ravera, M. Jaye, P.J. Donohue, J.A. Winkles. *J. Cell Biol.* **111**, 2129 (1990).

FIGURE 1

Rat marrow stromal cell CFU mineralization assay

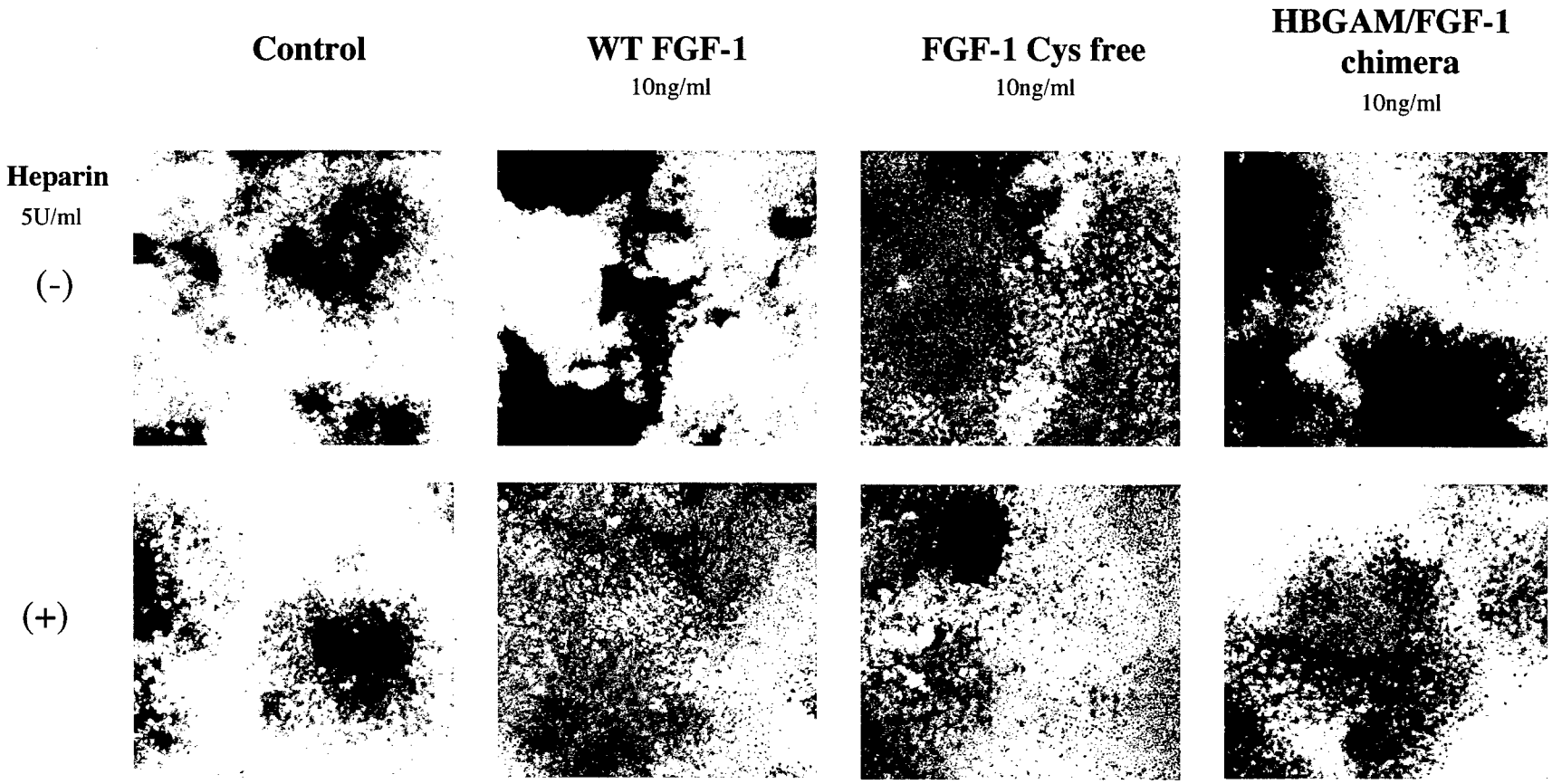

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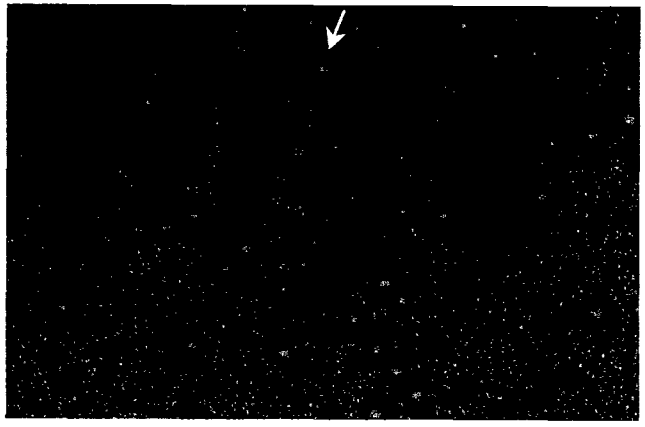
FIGURE 2

A.



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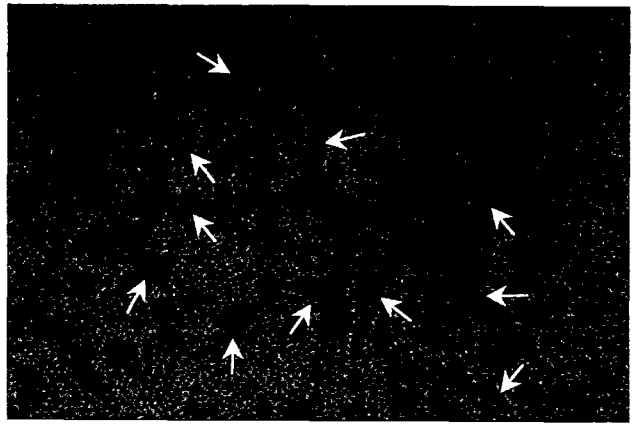
C.



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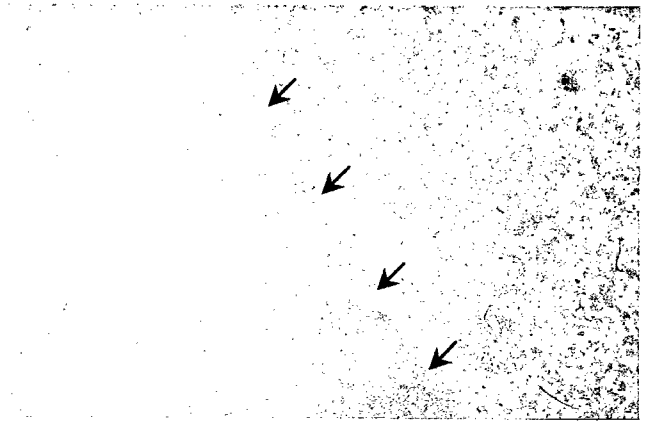
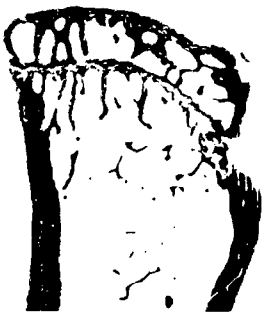


NR 1

OVX 2



WT 20

CHIM 20

CF 20

678 20

FIGURE 3

FIGURE 4

Effects of FGF-1 and Cys-Free Mutant on Systolic BP (5ug/kg)

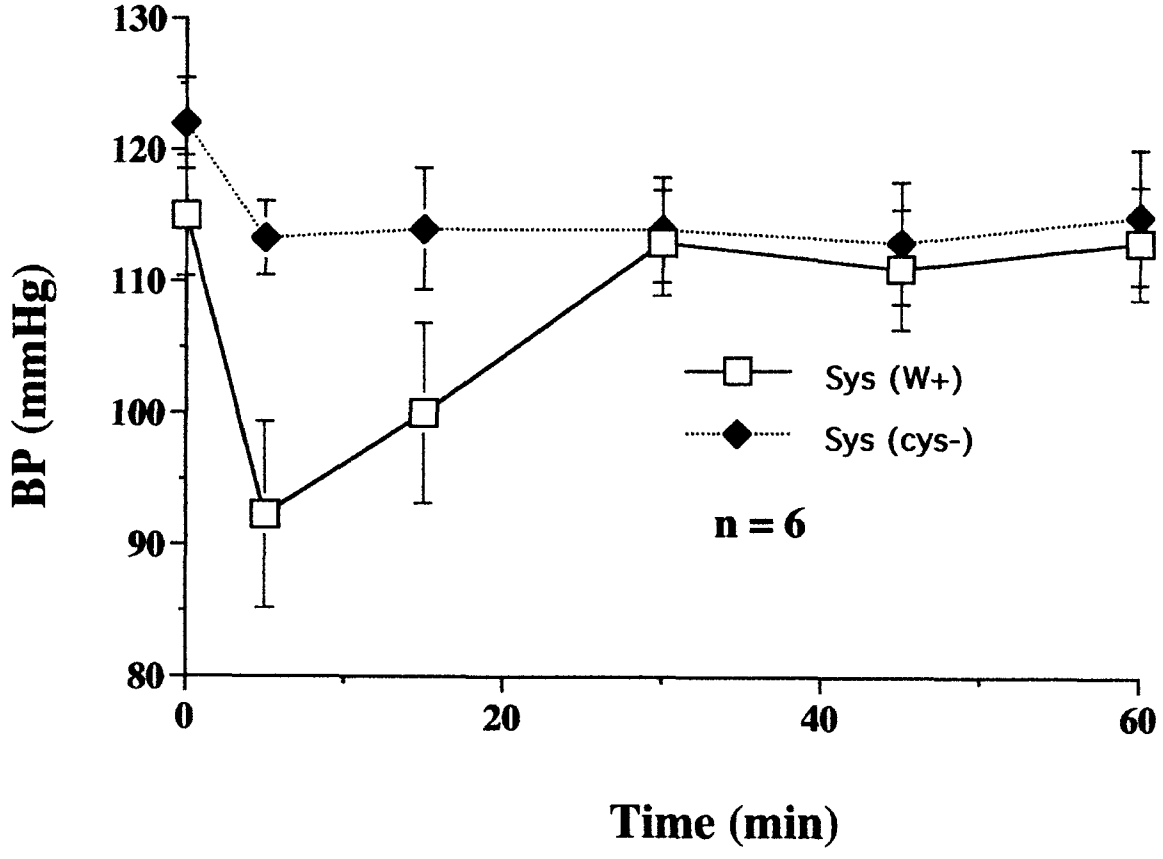
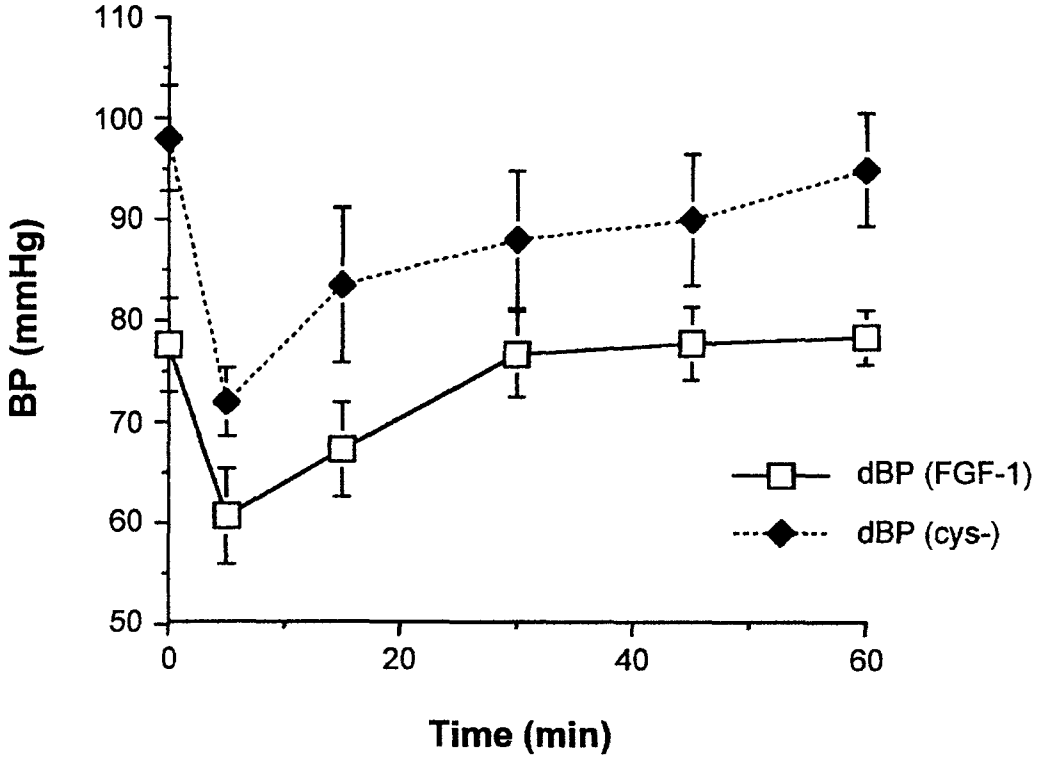


FIGURE 5

Effects of FGF-1 and Cys-Free Mutant on Diastolic BP (30ug/kg)



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